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1. Your reference

89024/JND

2. Patent application number

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17 NOV 1998 9825215.8

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

Cambridge Molecular Technologies Limited
Unit 3, Cambridge Techno Park
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Patents ADP number (*if you know it*)

7541170001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

ISOLATING NUCLEIC ACID

5. Name of your agent (*if you have one*)

Page White & Farrer

"Address for service" in the United Kingdom to which all correspondence should be sent (*including the postcode*)

54 Doughty Street
London WC1N 2LS

Patents ADP number (*if you know it*)

1255003

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Country

Priority application number
(*if you know it*)Date of filing
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

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- a) any applicant named in part 3 is not an inventor, or
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Priority documents	N/R
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Request for substantive examination (Patents Form 10/77)	-
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11. I/We request the grant of a patent on the basis of this application.

Signature *Page White & Farrer* Date 17 November 1998
PAGE WHITE & FARRER

12. Name and daytime telephone number of person to contact in the United Kingdom Mr J N Daniels 0171 831 7929

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ISOLATING NUCLEIC ACID

The present invention relates to a method for isolating nucleic acid, and particularly to a method for isolating plasmid DNA from a plasmid DNA-containing material.

Conventional procedures for the purification of nucleic acid, such as DNA, generally require multiple steps including lysis of source material followed by fractionation steps which may involve column chromatography. Where DNA manipulation is to be carried out, small scale DNA preparations are required routinely, often in large quantities for the purpose of screening DNA from the source cells. These processes are time consuming and labour intensive.

Various methods have been proposed in the purification of such DNA, including a precipitation method in EP-A-0376080, an ultrafiltration method in WO-A-87/07645 and EP-A-0517515 and cationic exchange resins in EP-A-0281390 and EP-A-0366438. A simplified method involving a filter, which is automatable, is disclosed in WO-A-95/02049.

Each of these methods suffers from a disadvantage that a series of steps is required and/or special apparatus is required to achieve sufficient purification of the plasmid DNA. A need therefore arises for a much simpler method involving readily-available apparatus and relatively inexpensive reagents. In a known approach for rapid purification of genomic DNA, RNA or protein, a mixture of phenol, chloroform and guanidine is used (Chomczynski, P. and Sacchi, N., 1987 *Anal Biochem.* 162: 156; Chomczynski, P., 1993 *Biotechniques* 15: 532) in which the DNA is extracted into an aqueous phase. This method is unsuitable for isolating plasmid DNA. Moreover, the use of phenol and chloroform is undesirable as these are toxic substances.

The present invention aims to overcome the disadvantages of the prior art and to provide a simplified method for isolating plasmid DNA.

Accordingly, the present invention provides a method for isolating plasmid DNA from DNA containing material which comprises plasmid DNA and genomic DNA, comprising:

- (i) extracting the plasmid DNA into a water-immiscible organic solvent capable of supporting plasmid DNA, by mixing the material with the organic solvent, a chaotrope and water under conditions to denature the genomic DNA; optionally separating the organic and aqueous phases of step (i); and
- (ii) recovering the plasmid DNA from the organic phase.

Accordingly, the present invention provides a "one step" method which is simple to perform and which requires no specialised laboratory apparatus. It is surprisingly found that this method is capable of extracting plasmid DNA to high purity and with particularly low or zero contamination from genomic DNA which might be present in the plasmid DNA-containing material. In a preferred arrangement, the organic solvent is capable of selectively supporting the plasmid DNA with the exclusion of genomic DNA present in the plasmid DNA-containing material.

The method of the present invention may be performed on a small routine laboratory scale working with solution volumes of microlitres or millilitres. Alternatively, the method may be scaled up even to pilot or industrial scale involving volumes of litres or greater.

In extraction step (i), the DNA-containing material is mixed with the reagents under conditions to denature the genomic DNA typically whereby the plasmid DNA is partitioned into an organic

phase and the genomic DNA is partitioned into an aqueous phase. Such conditions include basic conditions or elevated temperature. Suitable elevated temperatures are of at least 65°C and more preferably in the range 70 to 95°C for a time sufficient to denature the plasmid DNA such as from about 30s to about 10mins, preferably around five minutes. Incubation times longer than about 10 minutes at elevated temperature should not adversely affect the plasmid DNA but are undesirable for using the organic solvent. In a preferred arrangement, basic conditions are used in which a base is present. The base is typically a hydroxide such as an alkali metal hydroxide, preferably sodium hydroxide. The base is preferably present at a concentration in the range 100mM to 200mM. Incubation time is usually in the range from about 30s to about 10mins, preferably around five minutes. Excessive incubation under basic conditions can damage the plasmid DNA.

Without wishing to be bound by theory, it is thought that differential solubility between plasmid and genomic DNA under denaturing condition may result in plasmid DNA in an undenatured or reversibly denatured state partitioning into the organic phase. In contrast denatured genomic DNA partitions into the aqueous phase.

The organic solvent must be immiscible with the aqueous phase and preferably comprises an alcohol which may be aliphatic or aromatic and which may be linear or branched chain. The alcohol is preferably a C₃ to C₆ alcohol, more preferably a C₄ to C₆ alcohol and most preferably comprises a butanol such as N-butanol.

The chaotrope may be any normally-recognised chaotrope and is preferably selected from guanidine hydrochloride, guanidine thiocyanate, sodium perchlorate and mixtures thereof. A preferred chaotrope is guanidine hydrochloride. Typically, the

chaotrope is present at a concentration in the range 0.7M to 1.2M, based on the combination of organic solvents, chaotrope water. The concentration of the chaotrope is preferably about 0.9M.

The amount of organic solvent is typically in the range from 20 to 70% based on the volume of the combination of organic solvent, chaotrope or water and is preferably in the range from 35 to 50%, more preferably around 42%.

The exact organic solvent, chaotrope, base and amounts thereof are readily determinable by routine experimentation. Each of these reagents may be mixed with the DNA-containing material in any order or may be premixed prior to addition to the plasmid-containing material. In a convenient arrangement, the organic solvent, chaotrope, base and water are combined to form an extraction mixture. In this arrangement, the extraction step (i) comprises mixing the extraction mixture with the DNA-containing material.

At laboratory scale, the step (ii) of separating the organic and aqueous phases may be conveniently carried out by allowing the phases to separate or encouraging separation on the basis of density by a short spin in a microcentrifuge. Typically, either the organic or aqueous phase is removed from the other prior to recovery step (iii). For example, the organic phase containing the plasmid DNA may be transferred from one container to another by pipette prior to recovery. On a larger scale, removal of one phase from the other could be performed by any conventional method including pumping or running off by gravity one of the two phases.

In one arrangement, recovery step (iii) includes precipitation of the plasmid DNA from the organic solvent. For example, the DNA-containing organic phase may be mixed with a precipitating

agent that can precipitate the plasmid DNA from the organic solvent and the precipitated plasmid DNA is separated from the solvent. The precipitated plasmid DNA may also be washed in a washing step. The precipitating agent may comprise an alcohol such as ethanol and may further comprise an acetate salt such as sodium acetate.

The DNA-containing material may comprise any known DNA-containing material such as a bacterial culture which may be lysed or unlysed.

In a further aspect, the present invention provides an extraction mixture for selectively extracting plasmid DNA from a DNA-containing material, which extraction mixture comprises a water-immiscible organic solvent capable of supporting plasmid DNA, a chaotrope and water. The extraction mixture preferably further comprises a base.

The organic solvent, chaotrope, base and amounts thereof are typically those described above.

The present invention will now be described in further detail, by way of example only, with reference to the following Examples.

Example 1

General procedure

Bacterial culture (*E. coli* containing pBluescript; 0.5ml) was spun down in an eppendorf tube using a microcentrifuge and the supernatant was discarded. The pellet was resuspended in TE buffer (tris[hydroxymethyl]aminomethane hydrochloride 10mM, EDTA 1mM; pH8.0; 200 μ l) to form a resuspended pellet containing both genomic and plasmid DNA. An extraction mixture was selected according to the Table below, mixed very well and 0.5ml thereof was added to the resuspended pellet and gently mixed. The eppendorf containing the mixture was spun in a microcentrifuge

for 30 seconds to yield two phases; an upper organic phase and a lower aqueous phase. The organic phase was removed carefully to a fresh eppendorf tube avoiding any contaminating debris. Following measurement of the volume of the removed organic phase, sodium acetate (0.1vols; 3M) and ethanol were added (2vols) to precipitate the plasmid DNA. The eppendorf was spun in a microcentrifuge for 20 minutes and the ethanol supernatant removed. The pellet was rinsed with fresh ethanol (70%; 200 μ l) and spun for 5 minutes. The ethanol was removed and the pellet dried and resuspended in water (20 μ l). The resultant plasmid-containing DNA solution could then be assayed by visualisation on an agarose gel and the amount of DNA determined quantitatively by spectrophotometry or by fluorescence.

Table of Extraction Mixtures Tested

<u>CHAOTROPE</u>	<u>NaOH</u>	<u>SOLVENT</u>	<u>PLASMID DNA RECOVERY</u>
GuSCN 0.9M	150mM	N-Butanol 42%	Poor
GuSCN 0.9M	90mM	N-Butanol 42%	Poor
GuSCN 0.9M	200mM	N-Butanol 42%	Poor
GuSCN 0.9M	90mM	N-Butanol 20%	Poor
GuSCN 0.9M	150mM	N-Butanol 20%	Poor
GuSCN 0.9M	200mM	N-Butanol 20%	Good
GuSCN 0.9M	90mM	N-Butanol 70%	No
GuSCN 0.9M	150mM	N-Butanol 70%	No
GuSCN 0.9M	200mM	N-Butanol 70%	No
GuHCl 0.9M	90mM	N-Butanol 42%	Good
GuHCl 0.9M	150mM	N-Butanol 42%	Good
GuHCl 0.9M	200mM	N-Butanol 42%	Good
GuHCl 0.9M	90mM	N-Butanol 20%	OK
GuHCl 0.9M	150mM	N-Butanol 20%	OK
GuHCl 0.9M	200mM	N-Butanol 20%	Poor
GuHCl 0.9M	90mM	N-Butanol 70%	OK
GuHCl 0.9M	150mM	N-Butanol 70%	OK
GuHCl 0.9M	200mM	N-Butanol 70%	Poor
GuHCl 0.9M	90mM	2 methyl propanol 20%	Poor
GuHCl 0.9M	150mM	2 methyl propanol 20%	Poor
GuHCl 0.9M	200mM	2 methyl propanol 20%	Poor
GuHCl 0.9M	90mM	2 methyl propanol 70%	No
GuHCl 0.9M	150mM	2 methyl propanol 70%	No
GuHCl 0.9M	200mM	2 methyl propanol 70%	No
GuHCl 0.9M	90mM	2 methyl propanol 42%	Poor
GuHCl 0.9M	150mM	2 methyl propanol 42%	OK
GuHCl 0.9M	200mM	2 methyl propanol 42%	OK

GuHCl 0.9M	90mM	Butan-2-ol	42%	Poor
GuHCl 0.9M	150mM	Butan-2-ol	42%	OK
GuHCl 0.9M	200mM	Butan-2-ol	42%	Good
GuHCl 0.9M	90mM	Butan-2-ol	20%	Poor
GuHCl 0.9M	150mM	Butan-2-ol	20%	Poor
GuHCl 0.9M	200mM	Butan-2-ol	20%	Poor
Na Perchlorate 0.9M	90mM	N-Butanol	42%	Poor
Na Perchlorate 0.9M	150mM	N-Butanol	42%	Poor
Na Perchlorate 0.9M	200mM	N-Butanol	42%	Poor
Na Perchlorate 0.9M	90mM	N-Butanol	70%	Poor
Na Perchlorate 0.9M	150mM	N-Butanol	70%	Poor
Na Perchlorate 0.9M	200mM	N-Butanol	70%	Poor
Na Perchlorate 0.9M	90mM	N-Butanol	20%	Poor
Na Perchlorate 0.9M	150mM	N-Butanol	20%	Poor
Na Perchlorate 0.9M	200mM	N-Butanol	20%	Poor
Na Perchlorate 0.9M	200mM	2 methyl propanol	20%	OK
Na Perchlorate 0.9M	90mM	2 methyl propanol	70%	OK
Na Perchlorate 0.9M	150mM	2 methyl propanol	70%	Poor
Na Perchlorate 0.9M	200mM	2 methyl propanol	70%	Poor
Na Perchlorate 0.9M	90mM	Butan-2-ol	42%	Poor
Na Perchlorate 0.9M	150mM	Butan-2-ol	42%	Poor
Na Perchlorate 0.9M	200mM	Butan-2-ol	42%	OK
Na Perchlorate 0.9M	90mM	Butan-2-ol	20%	No
Na Perchlorate 0.9M	150mM	Butan-2-ol	20%	No
Na Perchlorate 0.9M	200mM	Butan-2-ol	20%	No

Good Approximately 1 μ g DNA recovery
OK Approximately 200ng DNA recovery
Poor Just visible on agarose gel electrophoresis

It may be concluded from these results that each recognised chaotrope works and that the guanidine hydrochloride is preferred over the guanidine thiocyanate which is, in turn, preferred over sodium perchlorate in terms of DNA recovery. As to solvents, butanol was found to work best whereas pentanol gave only poor DNA recovery. Ethanol and isopropanol were found not to be water-immiscible. Of the butanols, N-butanol was found to be better than either butan-2-ol or 2 methyl propanol.

Whilst TE was used as the resuspension buffer in the procedure, water could also be used, as well as other resuspension buffers.

Example 2

General procedure for extraction using heat instead of alkaline pH

Bacterial culture (*E. coli* containing pBluescript; 0.5ml) was spun down in an eppendorf tube using a microcentrifuge and the supernatant was discarded. The pellet was resuspended in TE buffer (tris[hydroxymethyl]aminomethane hydrochloride 10mM, EDTA 1mM; pH8.0; 200 μ l) to form a resuspended pellet containing both genomic and plasmid DNA. An extraction mixture was selected according to the Table below, mixed very well and 0.5ml was added to resuspended pellet and gently mixed. The eppendorf tube was then placed in a hot water bath at a temperature in the range 70 to 95°C for five minutes and the contents frequently mixed. Care was taken with the lid of the eppendorf tube because of solvent expansion in the tube. The tube was then rapidly cooled on ice for three minutes, which had the effect of separating the plasmid and genomic DNA. The eppendorf containing the mixture was spun in a microcentrifuge for 30 seconds to yield two phases; an upper organic phase and a lower aqueous phase. The organic phase was removed carefully to a fresh eppendorf tube avoiding any contaminating debris. Following measurement of the volume of the removed organic phase, sodium acetate (0.1vols; 3M) and ethanol were added (2vols) to precipitate the plasmid DNA. The eppendorf was spun in a microcentrifuge for 20 minutes and the ethanol supernatant removed. The pellet was rinsed with fresh ethanol (70%; 200 μ l) and spun for 5 minutes. The ethanol was removed and the pellet dried and resuspended in water (20 μ l). The resultant plasmid-containing DNA solution could then be assayed by visualisation on an agarose gel and the amount of DNA determined quantitatively by spectrophotometry or fluorescence.

Results comparable to those of Example 1 were obtained although yields were slightly lower and minor contamination with genomic DNA was observed.

CLAIMS:

1. A method for isolating plasmid DNA from a DNA containing material which comprises plasmid DNA and genomic DNA, comprising:

(i) extracting the plasmid DNA into a water-immiscible organic solvent capable of supporting plasmid DNA, by mixing the material with the organic solvent, a chaotrope and water under conditions to denature the genomic DNA; and

(ii) recovering the plasmid DNA from the organic phase.

2. A method as claimed in claim 1, wherein the organic solvent is capable of selectively supporting the plasmid DNA to the exclusion of denatured genomic DNA.

3. A method as claimed in claim 1 or claim 2, wherein the conditions to denature the plasmid DNA comprise basic conditions or a temperature of at least 65°C.

4. The method as claimed in claim 3, wherein the conditions to denature the plasmid DNA comprise basic conditions in which a base is present.

5. A method as claimed in claim 4, wherein the organic solvent comprises a C₃ to C₆ alcohol.

6. A method as claimed in claim 5, wherein the C₃ to C₆ alcohol comprises butanol.

7. A method as claimed in claim 6, wherein the butanol comprises n-butanol.

8. A method as claimed in any one of claims 4 to 7, wherein the chaotrope is selected from the group consisting of guanidine

hydrochloride, guanidine thiocyanate, sodium perchlorate and mixtures thereof.

9. A method as claimed in claim 8, wherein the chaotrope comprises guanidine hydrochloride.

10. A method as claimed in any one of claims 4 to 9, wherein the base comprises a hydroxide.

11. A method as claimed in claim 10, wherein the hydroxide comprises sodium hydroxide.

12. A method as claimed in any one of claims 4 to 11, wherein the organic solvent, the chaotrope, the base and the water are combined to form an extraction mixture, and extraction step (i) comprises mixing the extraction mixture with the plasmid DNA-containing material.

13. A method as claimed in any one of claims 4 to 12, wherein the amount of organic solvent is in the range from 20 to 70% based on the volume of the combination of organic solvent, chaotrope and water.

14. A method as claimed in claim 13, wherein the amount of the organic solvent is in the range from 35 to 50%.

15. A method as claimed in claim 14, wherein the amount of the organic solvent is about 42%.

16. A method as claimed in any one of claims 4 to 15, wherein the chaotrope is present at a concentration of from 0.7M to 1.2M based on the combination of organic solvent, chaotrope and water.

17. A method as claimed in claim 16, wherein the concentration of the chaotrope is about 0.9M.

18. A method as claimed in any one of claims 4 to 17, wherein the recovery step (ii) comprises mixing the DNA-containing organic phase with a precipitating agent that can precipitate the plasmid DNA from the organic solvent, and separating the precipitated plasmid DNA from the solvent.

19. A method as claimed in claim 18, wherein the recovery step (ii) further comprises a washing step in which the precipitated plasmid DNA is washed.

20. A method as claimed in claim 18 or claim 19, wherein the precipitating agent comprises an alcohol.

21. A method as claimed in claim 20, wherein the alcohol comprises ethanol.

22. A method as claimed in any one of claims 18 to 21, wherein the precipitating agent further comprises an acetate salt.

23. A method as claimed in claim 22, wherein the acetate salt comprises sodium acetate.

24. A method as claimed in any one of the preceding claims, which further comprises a step of separating the organic and aqueous phases of step (i) prior to recovering the plasmid DNA.

25. A method as claimed in claim 24, wherein the step of separating the organic and aqueous phases further comprises centrifugation of the mixture formed in step (i) to facilitate separation of the mixture into the organic and aqueous phases.

26. A method as claimed in any one of the preceding claims, wherein the DNA-containing material comprises a lysed or unlysed bacterial culture.

27. An extraction mixture for selectively extracting plasmid DNA from a DNA-containing material, which extraction mixture comprises a water-immiscible organic solvent capable of supporting plasmid DNA, a chaotrope and water.

28. An extraction mixture as claimed in claim 27, which further comprises a base.

29. An extraction mixture as claimed in claim 28, wherein the base comprises a hydroxide.

30. An extraction mixture as claimed in claim 29, wherein the hydroxide comprises sodium hydroxide.

31. An extraction mixture as claimed in any one of claims 27 to 30, wherein the organic solvent is capable of selectively supporting plasmid DNA to the exclusion of genomic DNA.

32. An extraction mixture as claimed in any one of claims 27 to 31, wherein the organic solvent comprises a C₃ to C₆ alcohol.

33. An extraction mixture as claimed in any one of claims 27 to 32, wherein the organic solvent comprises butanol.

34. An extraction mixture as claimed in claim 33, wherein the butanol comprises n-butanol.

35. An extraction mixture as claimed in any one of claims 27 to 33, wherein the organic solvent constitutes from 20 to 70% based on the volume of the extraction mixture.

36. An extraction mixture as claimed in claim 35, wherein the organic solvent constitutes from 35 to 50 % of the extraction mixture.

37. An extraction mixture as claimed in claim 36, wherein the organic solvent constitutes about 42% of the extraction mixture.

38. An extraction mixture as claimed in any one of claims 27 to 37, wherein the chaotrope is selected from the group consisting of guanidine hydrochloride, guanidine thiocyanate, sodium perchlorate and mixtures thereof.

39. An extraction mixture as claimed in claim 38, wherein the chaotrope comprises guanidine hydrochloride.

40. An extraction mixture as claimed in any one of claims 27 to 39, wherein the concentration of chaotrope in the extraction mixture is from 0.7M to 1.2M.

41. An extraction mixture as claimed in claim 40, wherein the concentration of the chaotrope in the extraction mixture is about 0.9M.

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